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<p>(54) Title: NUCLEIC ACID SEQUENCE DETECTION</p> <p>(57) Abstract</p> <p>The present invention provides a detection system suitable for use in a method of detecting the presence or absence of a predetermined nucleic acid sequence in a sample of nucleic acid. The system comprises a first oligonucleotide primer capable of annealing with a first strand of nucleic acid containing said predetermined nucleic acid sequence, such that a 3' end region of the first primer anneals with a first portion of said predetermined nucleic acid sequence; a second oligonucleotide primer having a 3' end region capable of annealing with a second strand of nucleic acid complementary to said first strand of nucleic acid such that a 3' end region of the second primer anneals with a portion of said second strand which portion is complementary to a second portion of said predetermined nucleic acid sequence, which second portion is spaced from end 5' of said first portion. One of the first and the second primers is labelled and the other is anchored to a substrate. Upon subjecting a sample of nucleic acid to an amplification reaction in the presence of said first and second oligonucleotide primers the presence of said predetermined nucleic acid sequence is indicated by the production of an amplified labelled nucleic acid product captively retained on said substrate.</p>		

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NUCLEIC ACID SEQUENCE DETECTION

This invention relates to the detection of target nucleic
5 acid sequences.

There is now available a considerable body of information
on the sequences of nucleic acid which are of particular
significance to inter alia the health of animals including
10 inter alia the sequences of various viruses and the
sequences of particular mutations or polymorphisms (which
are more common mutations) in the genomic DNA of a human or
other animal. Thus it is in principle possible to detect
the presence of such viruses and mutations or polymorphisms
15 by the use of tests for the detection of target nucleic
acid sequences which are more or less characteristic of
said viruses and mutations or polymorphisms. Existing
tests are however relatively slow, cumbersome, and/or
expensive and thus have been of limited or restricted use.

20

In more detail, it has been increasingly recognised in
recent years that mutations in genomic DNA which can be as
small as a single base or base pair, can give rise to
serious illnesses. Given though that a single gene can
25 contain thousands of bases, it can be difficult to screen
for such small changes.

One example of such a polymorphism occurs in the gene
encoding clotting factor V. A single mutation (G to A at
30 base 1691), replaces arginine 506 with glutamine, and
results in a clotting factor (Factor V leiden) which is
resistant to inactivation by activated Protein C, and
therefore more thrombogenic (Dahlback et al 1993, Bertina
et al 1984). This polymorphism is present in 2-5% of the
35 normal population, but in patients with venous thrombosis
rises to 15-20%, and thus appears to be a major risk factor
for venous thrombosis.

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There are many techniques available for the detection of mutation which vary greatly in their complexity and sensitivity. One of the major breakthroughs in this field was the development of ARMS PCR (amplification refractory mutation System; also known as SSP-PCR: sequence specific primer). While this technique has greatly accelerated the process of mutation screening, it still relies on the relatively cumbersome and time consuming process of electrophoresis to detect differences in electrophoretic mobility associated with the difference in nucleic acid sequence. Electrophoresis is a relatively cumbersome and time consuming procedure requiring the use of expensive materials and specialised apparatus under laboratory conditions which results in significant delays in obtaining a diagnosis.

A particularly significant problem in relation to virus detection, arises in relation to blood and blood products where viral contamination e.g. with Hepatitis A, B and/or C, Human Immunodeficiency Virus (HIV), Parvo Virus B-19, of blood donations can pose serious risks to the recipients of the blood transfusions or blood products. Existing screening methods are largely based on the detection of antibodies to the viral contaminant, in the blood. In practice, though, detectable levels of antibody to virus concerned may take several weeks to develop in the donor so that a blood donation from a recently infected donor cannot be successfully detected in this way. DNA or RNA detection can be used for such samples, however the large number of samples requiring to be analysed from even small blood donor centres places huge demands on any electrophoresis system.

It is an object of the present invention to avoid or minimise one or more of the above disadvantages.

The present invention provides a detection system suitable for use in a method of detecting the presence or absence of

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- a predetermined nucleic acid sequence in a sample of nucleic acid, the system comprising:
- a first oligonucleotide primer capable of annealing with a first strand of nucleic acid containing said predetermined nucleic acid sequence, such that a 3' end region of the first primer anneals with a first portion of said predetermined nucleic acid sequence;
- a second oligonucleotide primer having a 3' end region capable of annealing with a second strand of nucleic acid complementary to said first strand of nucleic acid such that a 3' end region of the second primer anneals with a portion of said second strand which portion is complementary to a second portion of said predetermined nucleic acid sequence, which second portion is spaced from and 5' of said first portion; and
- one of the first and the second primers being labelled with the other of said first and said second primers being anchored to a substrate, whereby upon subjecting a sample of nucleic acid to an amplification reaction in the presence of said first and second oligonucleotide primers the presence of said predetermined nucleic acid sequence is indicated by the production of an amplified labelled nucleic acid product captively retained on said substrate.
- In a further aspect the present invention provides a method of detecting the presence or absence of a predetermined nucleic acid sequence in a sample of nucleic acid, which method comprises the steps of:
- providing a first oligonucleotide primer capable of annealing with a first strand of nucleic acid containing said predetermined nucleic acid sequence, such that a 3' end region of the first primer anneals with a first portion of said predetermined nucleic acid sequence;
- a second oligonucleotide primer having a 3' end region capable of annealing with a second strand of nucleic acid complementary to said first strand of nucleic acid such that a 3' end region of the second primer anneals with a portion of said second strand which portion is

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complementary to a second portion of said predetermined nucleic acid sequence, which second portion is spaced from and 5' of said first portion; and
one of the first and the second primers being labelled with
5 the other of said first and said second primers being anchored to a substrate;
subjecting a sample of nucleic acid comprising the first and, complementary, second strands to an amplification reaction in the presence of said first and second
10 oligonucleotide primers;
removing unreacted labelled primer; and
detecting the presence of amplified labelled nucleic acid product captively anchored on said substrate.

15 Thus by means of the present invention it is possible to provide a highly sensitive detection of nucleic acid sequences of interest for one reason or another - for example to detect the presence of particular viral pathogens or contaminants, particular foreign nucleic
20 acids, or particular genomic DNA mutations or polymorphisms.

The present invention can also be used to detect the absence of or freedom from particular nucleic acid
25 sequences, whether this be from particular micro-organisms such as viral pathogens or contaminants, particular foreign nucleic acids, or particular normal or mutated genomic DNA sequences. It will be appreciated though that it may be desirable to seek positive verification of any essentially
30 negative finding such as the absence of a particular mutation, by positive detection of the normal sequence or of another mutation.

It will be appreciated that whilst it is necessary to have
35 substantially detailed knowledge of the sequence of the first and second portions of the predetermined nucleic acid sequence in order to select suitable oligonucleotide primers, it is not, in principle, necessary to have such detailed knowledge of the sequence between these.

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Accordingly, in its broadest sense the expression "predetermined nucleic acid sequence" indicates merely that at least said first and second portions have a particular known sequence.

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Where it is desired to obtain positive detection of individual ones of two or more different nucleic acid sequences, then this may be carried out by carrying out separate tests and amplifications with different combinations of primers containing the respective labelled primers. In a preferred form of the invention though two or more such primer combinations are used together simultaneously but with different labels in the labelled primers so that the presence of either or both of the different nucleic acid sequences may be indicated by detection of the respective labels. Various labelling systems are available which allow the selective identification of one label in the presence of another label, such as for example fluorescent labels which fluoresce selectively at different exciting wavelengths as further discussed hereinbelow. In this way it is possible to simultaneously test for a normal sequence and a particular mutation, two or more different mutations, two or more viral pathogens etc., thereby considerably improving the speed and/or efficiency of screening. It will of course be appreciated that where it is merely sufficient to know that any one of a plurality of predetermined nucleic acid sequences (e.g. corresponding to a number of different viruses) is present, then the different combinations of primers may be used together in the same reaction zone without the need for mutually distinguishable labels.

The present invention may be used with various nucleic acids including genomic, double stranded, DNA when screening for mutations. The invention may also be used with other double stranded nucleic acids e.g. an RNA - cDNA duplex, as well as with a single stranded nucleic acid e.g. RNA. In the latter case it will be appreciated that the

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s cond, complementary, strand required for exponential amplification reaction may simply be produced automatically in the first cycle of the amplification reaction by extension of the first oligonucleotide primer.

- 5 Alternatively though the second, complementary, strand could be produced by transcription of the single stranded nucleic acid by any suitable means known in the art as a preliminary step.
- 10 It will also be appreciated that nucleic acid amplification reactions are normally carried out as a plurality, preferably, a multiplicity, of cycles, each of which substantially doubles the amount of amplified nucleic acid product previously obtained (so long as excess primers are
- 15 still available). The number of cycles used will depend on inter alia the initial concentration of the nucleic acid of interest and the sensitivity of the label detection system used and hence the concentration of amplified nucleic acid product required. In general there would normally be used
- 20 at least 15 amplification cycles, preferably from 25 to 35, advantageously from 20 to 40, amplification cycles.

The present invention provides in another aspect a method of detecting a target polynucleotide sequence at a

25 predetermined site in a nucleic acid sequence, which method comprises the steps of:

- providing:
- a sample containing said nucleic acid sequence in a double-stranded form having complementary first and second
- 30 strands;
- a first oligonucleotide primer having a 5' end and a 3' end and capable of hybridizing with said target sequence, with the 3' - end of said first oligonucleotide primer hybridized to said predetermined site, or to one side
- 35 thereof in the 5' to 3' direction of said sequence, on the first strand; and
- a second oligonucleotide primer capable of hybridizing with the second complementary strand with the 3' end of said

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- second oligonucleotide primer hybridized to said complementary strand at a site spaced from said predetermined site in the 3' to 5' direction of said first strand,
- 5 one of:
- a) the first and (b) the second oligonucleotide primers being labelled;
- anchoring the other of: said first and said second oligonucleotide primers to a substrate;
- 10 subjecting the double stranded nucleic acid sequence sample to a replication reaction in the presence of said first and second oligonucleotide primers, for providing replicated nucleic acid produced by extension of said labelled oligonucleotide primer, hybridized to a replicated nucleic
- 15 acid sequence produced by extension of said oligonucleotide primer anchored to said substrate, so as to be captively retained on said substrate;
- removing unreacted labelled oligonucleotide; and
- detecting the presence of any labelled material on said
- 20 substrate.

It will of course be understood that the method of the present invention is equally applicable whether the first primer is designed to hybridize with the sense or anti-

25 sense strand of the nucleic acid, and the second primer with the anti-sense or sense strand, respectively.

Accordingly unless otherwise expressly indicated or required by the context, it should be understood that any references herein to one or other forms of the nucleic acid

30 sequence (sense and anti-sense) in the methods and products of the inventions, also includes the other. It should also be noted that references herein to an oligonucleotide primer being capable of annealing or hybridizing with a nucleic acid sequence or strand in relation to the new

35 methods of the present invention are used to indicate the primer binding to a first part of said nucleic acid sequence or strand to a degree which is sufficient to permit replication of a second part of said nucleic acid sequence or strand extending from said first part in the 3'

to 5' direction of said nucleic acid sequence or strand by extension of the primer in a suitable replication reaction. As is already known in the art, the degree of correspondence or complementarity of the oligonucleotide primer sequence to said first part of the nucleic acid sequence or strand (i.e. matching of the bases in the primer to the bases in the nucleic acid sequence or strand) required to achieve may vary considerably ranging from 100% down to 50% or less depending on various factors such as length of the oligonucleotide primer, position of any mismatches especially with reference to proximity to the 3' end, and stringency of the hybridizing conditions used. Thus in general a greater degree of mismatch may be tolerated the longer the oligonucleotide primer and the more remote the mismatch(es) from the 3' end. Also a greater degree of mismatch may be tolerated when less stringent hybridizing conditions are used.

The present invention can be used for detecting various kinds of mutations including point mutations or single base changes, and mutations involving two or more adjacent base changes, as well as deletions or insertions of various lengths, at predetermined sites in predetermined nucleic acid sequences. It may also be noted that the precise alignment of the 3' end of the first oligonucleotide primer with the predetermined site of the mutation within the predetermined nucleic acid sequence, can be varied somewhat. Preferably the 3' terminal base of the first oligonucleotide primer, itself coincides with the mutation site i.e. the predetermined site contains the 5'-terminal base of the first portion. It is also possible though for the 3' terminal base to be aligned with the predetermined nucleic acid sequence so as to be slightly offset, e.g. by 1 to 3 bases, 5' of the mutation site so that another part of the 3' end region e.g. the second or third base from the 3' terminal base is aligned with the predetermined site. In another form of the invention the 3' terminal base is aligned with the nucleic acid sequence so as to be offset 3' from the mutation site. In this case it will be

appreciated that the same first oligonucleotide primer will bind to both normal and mutant forms and it is accordingly necessary to discriminate these other than on the basis of differences between normal and mutant form primers. Where
5 the mutation is in the form of a substantial deletion e.g. more than 500 bases then amplification could be designed in such a way with reference to reaction and cycling conditions that replication would not normally proceed successfully with the normal form and this can accordingly
10 provide a suitable basis for discrimination with presence of bound labelled material only in the presence of the mutation. Where the mutation coincides with a restriction site (in the normal or mutant form of the nucleic acid sequence then digestion of the replicated material
15 containing the labelled oligonucleotide with a suitable restriction enzyme for said restriction site can be used to "cut free" replicated material containing the labelled oligonucleotide, only in the case where the replicated material also contains the restriction site so that bound
20 label is only detected in the presence of the nucleic acid sequence form in which the restriction site is absent.

The present invention can also be used for detecting a wide range of predetermined nucleic acid sequences for a variety
25 of different purposes other than the detection of mutations. In particular the invention can be used for detecting the presence or absence of infections of particular microorganisms in various materials such as blood, blood products, food, plant material through the
30 detection of particular nucleic acids which may be viral DNA or RNA, genomic DNA or RNA from microorganisms such as bacteria, fungi etc. It will of course be appreciated that such materials may require a greater or lesser degree of pre-treatment prior to application of the methods of the
35 present invention, for example in order to release the nucleic acid sequences into solution in a form which will allow amplification. Thus, for example, in the case of blood this would normally be treated by at least heating to

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temperatures in excess of 90°C to inactivate proteases prior to the addition of enzyme solutions.

The choice of oligonucleotide primers in such cases will generally involve a significantly greater degree of freedom than in those cases concerned with the detection of particular mutations and will also depend on the degree of specificity required in any given case - for example, whether it is required to detect a member of a particular species or sub-species, or merely a member of some wider grouping such as genera, families, orders etc. In general the oligonucleotide primers will be selected in relation to a predetermined nucleic acid sequence from a region which is substantially non-variable and well conserved within the grouping concerned (although it will be appreciated that, in principle, assuming there is no excessive variation inbetween them, it is only necessary for the first and second portions of the predetermined nucleic acid sequence to be well conserved). Suitable regions are well known in the art for various infectious organisms. Thus for example in the case of blood, hepatitis C infections are of great concern and suitable nucleic acid sequences for use in the detection of such infections include the highly conserved 5' noncoding region of the viral genome (Garson et al 1991).

It will further be understood that the methods of the present invention may be carried out in various ways. In the case of a specific localised mutation such as a single base substitution or deletion, then the first oligonucleotide primer is preferably selected so that its 3' end "corresponds" (i.e. when the first oligonucleotide hybridizes with the first strand, its 3' end is opposite) to the mutation site. In the case of a relatively large deletion then there would need to be used a normal first oligonucleotide primer with its 3' end in proximity to the 3' end of the deleted sequence to bring it sufficiently close in the nucleic acid sequence to the second oligonucleotide to allow replication of a normal DNA

fragment. On the other hand a mutant first oligonucleotide more or less close to the 3' side of the predetermined site deletion could allow replication to proceed with a mutant deleted nucleic acid sample even where the spacing would be
5 too large to allow replication of the corresponding normal nucleic acid sequence with the normal first oligonucleotide primer.

In one preferred aspect the present invention provides a
10 method of deleting a nucleic acid mutation or the like at a predetermined site in a double stranded nucleic acid sequence, which method comprises the steps of:
providing:

a first oligonucleotide primer having a 5' end and a 3'
15 end, at least part of the 3' end being capable of hybridizing to either the mutated sequence or the non-mutated sequence on a first strand and incapable of hybridizing to the other of said mutated sequence or non-mutated sequence on the first strand;
20 a second oligonucleotide primer capable of hybridizing with a second complementary strand with the 3' end of said second oligonucleotide primer being hybridized to said complementary strand at a site spaced from said predetermined site in the 3' to 5' direction of said first
25 strand,
one of:

a) the first and b) the second oligonucleotide primers being labelled;
anchoring the other of: said first and said second
30 oligonucleotide primers to a substrate;
subjecting a double stranded nucleic acid sequence fragment containing said predetermined site to a replication reaction in the presence of said first and second oligonucleotide primers, for providing replicated nucleic
35 acid produced by extension of said labelled oligonucleotide primer, hybridized to a replicated nucleic acid sequence produced by extension of said other oligonucleotide primer anchored to said substrate, so as to be captively retained on said substrate;

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removing unreacted labelled oligonucleotide primer; and detecting the presence of any labelled material on said substrate.

- 5 In a simple form of the invention there is only one, normal or mutant, first oligonucleotide to detect the presence or absence of the respective, normal or mutant, nucleic acid sequence. Preferably though, in order to reduce the risk of false negatives, then both normal and mutant forms of
10 the first oligonucleotide are used in separate reaction zones (e.g. wells). A normal or mutant sequence will then always give a positive reaction in one or other of the reaction zones (except where the sequence contains a different mutation at the predetermined site - in which
15 case further mutant first oligonucleotides would need to be used to obtain a positive indication of the presence of such further mutations).

- In one preferred form the invention simply uses a first
20 oligonucleotide primer capable of hybridizing with the nucleic acid sequence to the 3' side of the predetermined site of the nucleic acid mutation, and a second oligonucleotide primer capable of hybridizing with the complementary form sequence to the 3' side of the
25 predetermined site (in the complementary form sequence), whereby replication and detection of labelled replicated nucleic acid sequences can only be obtained with mutant sequences.

- 30 In another preferred form the present invention provides a method of detecting a nucleic acid mutation or the like at a predetermined site in a double stranded nucleic acid sequence, which method comprises the steps of:
providing:

- 35 at least one of
a) a normal form first oligonucleotide primer having a 5' end and a 3' end and capable of hybridizing with a normal or mutant form of said sequence, with the 3' end of said first oligonucleotide primer hybridized to said

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predetermined site or to one side thereof in the 5' to 3' direction of said sequence, on a first strand; and

b) a mutated form first oligonucleotide primer having a 5' end and a 3' end and capable of hybridizing with a mutated form of said sequence with the 3' end of said mutated first oligonucleotide primer hybridized to said predetermined site or to one side thereof in the 5' to 3' direction of said sequence, on said first strand; and

a second oligonucleotide primer capable of hybridizing with a second complementary strand with the 3' end of said oligonucleotide primer hybridized to said complementary strand at a site spaced from said predetermined site in the 3' to 5' direction of said first strand,

one of: a) the first and (b) the second oligonucleotide primers being labelled;

anchoring the other of: said first and said second oligonucleotide primers to a substrate;

carrying out replication of a double stranded nucleic acid sequence fragment containing said predetermined site to a replication reaction in the presence of said first and second oligonucleotide primers, for providing replicated nucleic acid produced by extension of said labelled oligonucleotide primer hybridized to a replicated nucleic acid sequence produced by extension of said other oligonucleotide primer anchored to said substrate, so as to be captively retained on said substrate;

removing unreacted labelled oligonucleotide; and detecting the presence of any labelled material on said substrate.

It would in principle be possible to anchor the second (common) oligonucleotide to the substrate and utilize different ones of the normal and mutant normal mutant first oligonucleotide in different reaction zones. In general though it is more convenient to anchor the first oligonucleotide(s) to the substrate (in different reaction zones) and use the same free (labelled) second oligonucleotide in all the reaction zones.

Thus, with the method of the present invention, it is possible to detect relatively small nucleic acid mutations - in some cases as small as one base - in a relatively simple, reliable, economic, and rapid manner using well established in vitro replication techniques such a PCR and monitoring for the presence or absence of labelled product.

A variety of labelling systems suitable for use in the method of the invention are well known in the art including radioactive labels such as P^{32} or P^{33} , fluorescent labels such as fluorescein or rhodamine and coloured dye label systems such as biotinylation followed by development with Streptavidin-Horseradish peroxidase conjugate and peroxidase substrate.

Thus in some cases, where, for example, a radioactive label is used, the presence of bound label may be detected with suitable apparatus such as a scintillation counter. In other cases there may require to be used a developer reagent system for "developing" the bound label into a form which can be detected by simple visual inspection and/or with the aid of suitable apparatus e.g. spectroscopic apparatus.

A variety of oligonucleotides may be used for detecting any desired sequence. Preferably there are used oligonucleotides having from 15 to 40, most preferably from 18 to 35 nucleotides, although in the case of the anchored oligonucleotide this should preferably have a length of at least 25 nucleotides to help avoid interference with replication as a result of steric hindrance. Whilst longer nucleotides could be used these are generally less preferred for economic reasons.

The separation of the second portion from the first portion along the predetermined nucleic acid sequence being replicated may also be varied considerably. In general the spacing may be from 50 to 1000 nucleotides, preferably from 100 to 500 nucleotides when testing for a specific well

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defined mutation such as a point mutation or well defined breakpoint. Although larger spacings may be employed these are generally less efficient and do not provide any additional benefits. Smaller spacings of less than 100
5 nucleotides have been shown to be more efficient for detecting low levels of nucleic acids (Garson et al 1991).

In the case of mutations in the form of substantial deletions, then, as noted above, as an alternative to using
10 a first oligonucleotide capable of hybridising to a predetermined site within said deletion, there may simply be used first and second oligonucleotides at either side of said deletion with an interval therebetween (in the normal sequence) larger than that which can be replicated under
15 the replicating conditions used, but which is small enough in the presence of the deletion to allow replication to proceed under said replicating conditions, whereby the presence of the deletion is indicated by the detection of the labelled replicated material, while the normal sequence
20 does not yield any labelled replicated material. Thus in such cases it will be appreciated that the preferred oligonucleotide primer spacing is defined with reference to the deleted form of the predetermined nucleic acid sequence rather than the normal form. Similar considerations apply
25 to cases where there are substantial insertions and it is desired to detect the presence of a specific insertion, the preferred oligonucleotide primer spacing is defined with reference to the inserted form of the predetermined nucleic acid sequence.

30 Various techniques suitable for use in anchoring the (first or second) oligonucleotide to the substrate may be used according to inter alia the kind of substrate used. Most conveniently the latter is a microtitre plate or other
35 multi-reaction zone apparatus made of a plastics material such as polyalkene, polyacrylate, polystyrene etc. In some cases the oligonucleotide may be anchored directly to the plastics material by means of chemical cross-linking (Staros et al 1986).

In other cases the oligonucleotide primer may be anchored indirectly with the aid of a specially coated or treated reaction zone surface. Thus for example there may be used
5 a substrate in the form of a plate coated with streptavidin and the oligonucleotide biotinylated and then bound to the streptavidin coated plate.

In order to obtain sufficient bound labelled material to
10 facilitate detection there is desirably used a replication method which readily provides a substantial degree of amplification. The well known Polymerase Chain Reaction is particularly suitable in this connection.

15 Other in vitro replication methods that could be used include the ligase chain reaction (Wiedmann et al 1994).

In order to reduce the risk of obtaining false negative results there is desirably used in accordance with the
20 present invention, at least one positive control sample of nucleic acid containing the predetermined nucleic acid sequence of interest for testing in parallel with the unknown sample. Various forms of multi-zone apparatus particularly convenient for use in parallel testing
25 including for example, microtitre plates which have an array of a multiplicity of reaction walls, are known in the art.

In a further aspect the present invention provides a
30 diagnostic kit suitable for use in a method of the present invention comprising:

at least one of:

a) a normal form first oligonucleotide primer having a 5' end and a 3' end and capable of hybridising with a normal
35 or mutant form of said sequence, with the 3' -end of said first oligonucleotide primer hybridised to said predetermined site or to one side thereof in the 5' to 3' direction of said sequence, on a first strand; and

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- b) a mutated form first oligonucleotide primer having a 5' end and a 3' end and capable of hybridising with a mutated form of said sequence with the 3' end of said mutated first oligonucleotide primer hybridised to said predetermined site or to one side thereof in the 5' to 3' direction of said sequence, on said first strand; and
a second oligonucleotide primer capable of hybridising with a second complementary strand with the 3' end of said oligonucleotide primer hybridised to said complementary strand at a site spaced from said predetermined site in the 3' to 5' direction of said first strand,
one of:
a) the first and (b) the second oligonucleotide being labelled,
and the other of: said first and second oligonucleotide primers being anchored to a substrate.

- The present invention also provides a diagnostic kit suitable for use in a method of the present invention comprising:-
a first oligonucleotide primer having a 5' end and a 3' end capable of hybridising with a normal or mutant form of said sequence, with the 3' end of said first oligonucleotide primer hybridised to said predetermined site, or to one side thereof in the 5' to 3' direction of said sequence, on a first strand; and
a second oligonucleotide primer capable of hybridising with a second complementary strand with the 3' end of said second oligonucleotide primer hybridised to said complementary strand at a site spaced from said predetermined site in the 3' to 5' direction of said first strand,
one of:
a) the first and (b) the second oligonucleotide primers being anchored to a substrate,
and the other of: said first and second oligonucleotide primers being labelled.

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Further preferred features and advantages of the invention will appear from the following detailed example given by way of illustration.

5 Example 1 - Preparation of Diagnostic Kit for use in the detection of G1691A polymorphisn in gene encoding coagulation factor V.

Background

10 The cDNA sequence for the gene encoding coagulation or clotting factor V has been published (R.J. Jenny et al) and is also listed in the EMBL database under EMBL Accession number M16967.

A. Preparation of labelled (first) oligonucleotides

15 A "normal" sequence specific downstream, oligonucleotide having the following sequence: 5'-AAAAGTACCTGTATTCCTT-3' and corresponding to base positions 1691 to 1701 of the complementary form of the abovementioned cDNA sequence and a further 8 bases of the genomic sequence coding for an
20 intron details of which are also included in the abovementioned publication (R.J. Jenny et al), was biotinylated during manufacture.

A "mutant" sequence specific oligonucleotide having a
25 sequence identical to the above "normal" one except for the 3' terminal base which was C in place of T corresponding to the G/A mutation present in the genomic DNA sequence at the 1691 base position in the abovementioned published cDNA sequence, was similarly
30 labelled.

B. Preparation of anchored (second) oligonucleotide

A known upstream oligonucleotide having the sequence:
5'-TGCCAGTGCTTAACAAGAC-3' corresponding to base positions
35 1581 to 1600 of the abovementioned cDNA sequence, was anchored to a 96-well microtiter plate by a carbodiimide-mediated coupling reaction in accordance with the following procedure.

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15 μ l of animated oligo solution (100 ng oligo) was added to each well of the plate. And diluted with an equal volume of 20 μ M MES (2-[N-Morpholino]ethanesulphonic acid), 20 μ M EDTA. 30 μ l of cross linking solution was then added to each well and the plates were incubated at 37°C preferably 50°C for 3 hours. After incubation the plates were washed twice in dd. (double distilled) H₂O and blotted dry before being air dried at 60°C for 1 hour. Plates thus prepared can be stored at 4°C for at least 6 months.

10

Cross-linking solution;

40 mg preferably 400 mg EDC 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl and 500 μ g Sulpho-NHS (N-Hydroxysulphosuccinimide) made up to 5 ml with ddH₂O.

15

Example 2 - Method of Detecting G1691A mutation in the gene encoding coagulation factor V

20 A. Replication of DNA fragment sample

Genomic DNA (100-500ng in 5 μ l ddH₂O) from each sample was added to duplicate wells on the microtitre plate of the diagnostic kit of Example 1. A separate master mix solution having the composition indicated below was then added to each of these duplicates, one containing "normal" first oligonucleotide, the other containing "mutant" first oligonucleotide. Each well was then overlaid with 20 μ l mineral oil and subjected to 30 rounds of PCR (Polymerase Chain Reaction) amplification using the conditions indicated below.

30

Master mix solution - volumes per well;

5 μ l 10 X PCR Buffer (Promega)

3 μ l dNTP solution (1.25 μ M each)

2 μ l MgCl₂ solution (25 μ M)

35 1 μ l oligo (100ng of appropriate first oligonucleotide)

Taq polymerase (2U)

H₂O to 55 μ l (preferably 50 μ l)

PCR Cycling conditions:

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94°C 15s

55°C 15s

72°C 30s

30 cycles followed by 2 min hold at 72°C

5

B. Detection of Replicated DNA

The plates were then washed once in buffer A and once in buffer B. Streptavidin-HRP (horseradish peroxidase) conjugate was then added to each well (150µl of a 50ng solution), and the plates incubated at 37°C for 15 min. Plates were then washed once in buffer B and once in buffer A. 150µl of Sigmafast OPD (commercially available peroxidase substrate) solution was then added to each well, and the colour allowed to develop in the dark for 15 min. The presence of a yellow/brown colour indicates the presence of PCR replicated DNA product, and hence the allele corresponding to that particular, normal or mutant, first oligonucleotide.

20 Buffer A

100µM Tris-HCl pH 7.5; 800 µM NaCl

Buffer B

Buffer A with 0.5% BSA

25

Streptavidin Horseradish-peroxidase conjugate
Sigma Chemical Company product number S-5512

Sigmafast OPD

30 Sigma Chemical Company product number P9187

Example 3 - Preparation of a screening kit for use in the
detection of Hepatitis C virus in plasma donations.

35 Background

Viral contamination of donated blood is a serious problem in transfusion medicine. Direct detection of viral genome is the only reliable way to screen for hepatitis C (HCV)

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viraemia. This is most efficiently done using two 'nested PCR' reactions. Nested PCR improves both the yield of product from very low template concentrations, and the specificity of the PCR reaction, reducing the possibility of false positive reactions. The first PCR reaction using the outer primers (PT1 and PT2) yields a product of 81 base pairs in size, the second PCR reaction using two primers (PT3 and PT4) specific to the sequence amplified by the first pair of primers and yielding a product of 60 base pairs. These primers are directed against the highly conserved 5' noncoding region of the viral genome, and will amplify all previously described genotypes of HCV including Types I, II and III so that a positive result will simply indicate that at least one HCV Type (which may be any Type) is present in the test sample, this being sufficient in cases where, for example, the purpose of the test is simply to check the safety of a blood donation. Of course where it is desired to detect the presence of one particular Type of HCV, then a different set of oligonucleotide primers would require to be selected. This can be readily effected by selecting primers from regions of the nucleic acid sequence which are specific to the Type concerned, using the published sequence information.

25 Primer sequences (from Garson et al 1991)

PT1 5'-CGTTAGTATGAGTGTCTGTC-3' (antisense outer)
PT2 5'-CGGTGTACTCACCGGTTCC-3' (sense outer)
PT3 5'-AGTGTCTGTCAGCCTCCAGG-3' (antisense inner)
PT4 5'-CGGTTCCGCAGACCACTATG-3' (sense inner)

30

A. Preparation of oligonucleotides

The oligonucleotide primers were obtained from a commercial producer and supplier of such primers (Oswel DNA Service, Southampton, England). Oligonucleotides PT1 and PT2 were used without modification. Oligonucleotide PT4 was biotinylated during manufacture. Oligonucleotide PT3 was anchored to a 96 well microtitre plate (Nucleolink, Nunc, Inc. Naperville, IL, USA) by carbodiimide-mediated coupling in accordance with the following procedure.

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75 μ l 10 μ M 1-methyl-imidazole (1-MeIm), pH 7.0, containing 100 ng/ well of PT3 was pipetted into each well. 25 μ l of 40 μ M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (freshly prepared in 10 μ M 1-MeIm) was then added to each well and the plate was incubated at 50 °C for 5 hours. The plate was then washed 5 times in 0.4N NaOH 0.25% Tween 20 at 50 °C and twice in ddH₂O before being blotted dry.

Example 4 - Method of detecting HCV in blood donations.

10

A. Reverse transcriptase and first round replication.

5 μ l plasma or RNA extracted from a test sample, or a positive control RNA sample, was added to each reaction well of an untreated 96 well plate. (When viral load is high, 5 μ l of plasma will contain detectable levels of template; at lower levels, some form of concentration either by centrifugation and resuspension in a smaller volume, or chemical extraction and precipitation may be required. As this test is based on the presence or absence of a PCR product, it is essential to include a positive control RNA sample to ensure the reaction conditions are working). 45 μ l of master mix 1 was added to each well, and overlaid with 20 μ l mineral oil. The plate was then subjected to the following temperature cycles.

25

Stage 1 1 cycle

95 °C for 2 min
70 °C for 30 min.

Stage 2 30 cycles

30 95 °C for 20 s
55 °C for 30 s.

Master mix 1 volume per well

10 μ l 5X buffer (250 μ M Bicine pH 8.2, 575
35 μ M KOH, 40% Glycerol)
3 μ l dNTP solution
5 μ l 25 μ M Mn(OAc)₂ solution

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3 μ l mix of oligos PT1 and PT2 (0.2 μ M each)
1 μ l rTth DNA polymerase (Perkin Elmer or
Promega)
23 μ l sterile ddH₂O

5

Note: rTth DNA polymerase also has reverse transcriptase activity which is used in stage 1 to produce DNA copies of the RNA.

B. Second round replication.

10 5 μ l of the PCR product generated in each well of A (above) was added to a well of the microtitre plate prepared in example 3 (above). 45 μ l of master mix 2 was added to each well, and overlaid with 20 μ l mineral oil. The plate was then subjected to the following temperature cycles.

15

30 cycles

95 °C for 20 s

55 °C for 30 s.

20 Master mix 2 volume per well

10 μ l 5X buffer (250 μ M Bicine pH8.2, 575

μ M KOH, 40% Glycerol)

3 μ l dNTP solution

5 μ l 25 μ M Mn(OAc)₂ solution

25 3 μ l mix of oligos PT3 and PT4 (0.02 μ M PT3 and 0.2 μ M each)

1 μ l rTth DNA polymerase (Perkin Elmer or Promega)

23 μ l sterile ddH₂O

30

C. Detection of replicated DNA

The same procedure and materials as described in Example 2B hereinabove were used. The presence of a yellow/brown

35 colour indicates the presence of PCR replicated DNA product, and hence of an HCV nucleic acid, thereby indicating viral contamination with Hepatitis C virus.

Example 5 - Preparation of a diagnostic kit for the detection of G20210A polymorphism in the prothrombin gene (coagulation factor II_{Leiden})

5

Background. This polymorphism was reported by Poort et al (1996) and is associated with increased risk of thrombosis. The following two examples comprise a diagnostic kit suitable for the detection of this polymorphism.

10

A. Preparation of anchored oligonucleotides.

A 'normal' sequence specific downstream oligonucleotide having the following sequence: ATAGCACTGGGAGCATTGAGGCTC and corresponding to positions 20233-20210 to nnnn of the above mentioned DNA sequence (numbered according to Poort et al) was synthesised with a 5' amino group.

15

A 'mutant' sequence specific downstream oligonucleotide having the following sequence: ATAGCACTGGGAGCATTGAGGCTT and corresponding to positions nnnn to 20233-20210 of the above mentioned DNA sequence (numbered according to Poort et al) was likewise synthesised with a 5' amino group.

20

The above mentioned oligonucleotides were anchored to separate wells of a 96 well microtitre plate using the same procedure as in Example 1.

25

B. Preparation of labelled oligonucleotide.

An upstream oligonucleotide having the sequence TCTAGAAACAGTTGCCTGGC corresponding to base positions 19889-19908 of the above mentioned sequence was biotinylated at the 5' end during manufacture.

30

Example 6 Method of detecting G20210A polymorphism in the prothrombin gene (coagulation factor II_{Leiden})

35

A. Replication of DNA fragment sample

To the microtitre plate prepared in Example 5A, genomic DNA (100-500ng) or whole blood (2-5 µl) from each sample was

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added to one well coated with the 'normal' oligonucleotide,
and one well coated with the 'mutant' oligonucleotide. 50
µl of a master mix solution having the composition
indicated below was then added to each well of the
5 microtitre plate. Each well was then overlaid with 20 µl
mineral oil and subjected to 30 rounds of PCR amplification
using the same procedures as in Example 2A except that in
the Master mix solution there is used in the 1 µl oligo:
(100ng labelled oligonucleotide (from Example 5B)) and the
10 H₂O was added to 50 µl.

B. Detection of Replicated DNA

The same procedure and materials as described in Example 2B
15 hereinabove were used. The presence of a yellow/brown
colour indicates the presence of PCR replicated DNA
product, and hence the allele corresponding to that
particular, normal or mutant, first oligonucleotide.

20

References

Bertina, RM, Koeleman BPC., Koster T., Rosendaal FR.,
Dirven RJ., et al (1994)
Mutation in blood coagulation factor V associated with
25 resistance to activated protein C. Nature 369; 64-67

Dahlback B., Carlsson M. And Svensson PJ. (1993) Familial
thrombophilia due to a previously unrecognised mechanism
characterised by poor anticoagulant response to activated
30 protein C; Prediction of a cofactor to activated protein C.
PNAS USA, 90: 1004-1008.

Staros JV., Wright RW & Swingle DM. (1986) Enhancement of
N-hydroxysulfosuccinimide of water-soluble carbodiimide-
35 mediated coupling reactions.

-26-

- Garson JA, Ring CJA and Tuke PW (1991) Improvement of HCV genome detection with short PCR products. Lancet 338;1466-1467.
- 5 Wiedmann M, Wilson WJ, Czaika J, Luo J, Barany F, Batt CA, (1994) Ligase chain reaction (LCR) - Overview and applications. PCR methods and Applications. 3, (4) S51-S64.
- Jenny RJ, Pittman DD, Toole JJ, Kriz RW, Aldape RA, Hewick
10 Rm, Kaufman RJ and Mann KG in 1987 (''Complete cDNA and derived amino acid sequence of human factor V'') Proc. Natl. Acad. Sci. USA 84:4846-4850.
- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM, (1996)
15 A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 88; 3698-3703.

CLAIMS

1. A detection system suitable for use in a method of detecting the presence or absence of a predetermined
5 nucleic acid sequence in a sample of nucleic acid, the system comprising:
a first oligonucleotide primer capable of annealing with a first strand of nucleic acid containing said predetermined nucleic acid sequence, such that a 3' end region of the
10 first primer anneals with a first portion of said predetermined nucleic acid sequence;
a second oligonucleotide primer having a 3' end region capable of annealing with a second strand of nucleic acid complementary to said first strand of nucleic acid such
15 that a 3' end region of the second primer anneals with a portion of said second strand which portion is complementary to a second portion of said predetermined nucleic acid sequence, which second portion is spaced from and 5' of said first portion; and
20 one of the first and the second primers being labelled with the other of said first and said second primers being anchored to a substrate, whereby upon subjecting a sample of nucleic acid to an amplification reaction in the presence of said first and second oligonucleotide primers
25 the presence of said predetermined nucleic acid sequence is indicated by the production of an amplified labelled nucleic acid product captively retained on said substrate.

2. A system according to claim 1 which includes a plurality
30 of different oligonucleotide primer combinations corresponding to different respective predetermined nucleic acid sequences.

3. A system according to claim 2 wherein said different
35 oligonucleotide primer combinations have different mutually distinguishable labels whereby the presence of any one or more of the plurality of different predetermined nucleic acid sequences may be detected at the same time in a sample.

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4. A system according to claim 2 wherein said different oligonucleotide primer combinations have the same label whereby the presence of any one or more of the plurality of
5 different predetermined nucleic acid sequences may be detected in alternate samples.

5. A system according to any one of claims 1 to 4 which includes a plurality of different oligonucleotide primer
10 combinations having a nested arrangement relative to each other with respect to a given predetermined nucleic acid sequence for use in a preliminary multi-stage nested amplification reaction.

15 6. A system according to any one of claims 1 to 5 which includes a nucleic acid amplification system.

7. A system according to claim 6 wherein said amplification system includes a polymerase which catalyses reverse
20 transcription of RNA and replication of DNA.

8. A system according to any one of claims 1 to 7 wherein each of said oligonucleotide primers has from 15 to 40 nucleotides.

25 9. A system according to any one of claims 1 to 8 wherein the anchored oligonucleotide primer(s) has (have) at least 25 nucleotides.

30 10. A system according to any one of claims 1 to 9 wherein the labeled oligonucleotide primer(s) has (have) a label selected from a radioactive label, a fluorescent label and a coloured dye label.

35 11. A detection system according to claim 1 which includes a developer reagent system for use in developing the label in any captively retained labelled nucleic acid, to facilitate detection of said label.

12. A system according to any of claims 1 to 11 wherein the substrate comprises a multi-reaction zone apparatus.

5 13. A system according to claim 12 wherein said apparatus comprises a microtitre plate of a plastics material.

14. A system according to claim 13 wherein the anchored primer is attached to the substrate by means of chemical
10 cross-linking.

15. A system according to claim 13 wherein the anchored primer is bound via a coating applied to a reaction zone surface.

15

16. A system according to any one of claims 1 to 15 which includes at least one positive control sample of nucleic acid, which sample contains said predetermined nucleic acid sequence(s).

20

17. A system according to any one of claims 1 to 16 for use in a method of detecting a mutation or polymorphism which system includes an oligonucleotide primer combination specific to the mutated or polymorphic form of the
25 predetermined nucleic acid sequence.

18. A system according to claim 17 which further includes an oligonucleotide primer combination specific to the normal form of the predetermined nucleic acid sequence.

30

19. A system according to claim 17 or claim 18 wherein said predetermined nucleic acid sequence includes base 1691 of the genomic DNA sequence encoding clotting factor V.

35 20. A system according to claim 19 when dependent on claim 18 wherein is included an oligonucleotide primer combination with two first and one second oligonucleotide primers having the sequences 5'-AAAAGTACCTGTATTCCTC-3' and

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5'-AAAAGTACCTGTATTCCTT-3', and 5'-TGCCCAGTGCTTAACAAGAC-3', respectively.

21. A system according to any one of claims 1 to 16 for use in method of detecting a predetermined nucleic acid from a viral DNA or RNA; or genomic DNA or RNA from a micro-organism or an RNA specifically associated therewith, which system includes an oligonucleotide primer combination substantially specific to said predetermined nucleic acid.

10

22. A system according to claim 21 wherein said predetermined nucleic acid is specific to a taxonomic grouping not smaller in size than a species, in which system said oligonucleotide primer is specific to a nucleic acid sequence which is substantially non-variable and well conserved within said taxonomic grouping.

15

23. A system according to claim 22 wherein said predetermined nucleic acid sequence is from a well conserved region of the HCV genome.

20

24. A system according to claim 23 wherein said predetermined nucleic acid sequence is from the highly conserved 5' noncoding region of the HCV viral genome.

25

25. A system according to claim 23 wherein the first and second primers are 5'-AGTGTCTGTCAGCCTCCAGG-3' and 5'-CGGTTCCGCAGACCACTATG-3'.

26. A system according to claim 24 wherein is used a second oligonucleotide primer combination for a preliminary stage of amplification of the nucleic acid sample in which the first and second primers are 5'-CGTTAGTATGAGTGTCTGTC-3' and 5'-CGGTGTACTCACCAGTTCC-3'.

30

27. A method of detecting the presence or absence of a predetermined nucleic acid sequence in a sample of nucleic acid, which method comprises the steps of:

35

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providing a first oligonucleotide primer capable of annealing with a first strand of nucleic acid containing said predetermined nucleic acid sequence, such that a 3' end region of the first primer anneals with a first portion of said predetermined nucleic acid sequence;
a second oligonucleotide primer having a 3' end region capable of annealing with a second strand of nucleic acid complementary to said first strand of nucleic acid such that a 3' end region of the second primer anneals with a portion of said second strand which portion is complementary to a second portion of said predetermined nucleic acid sequence, which second portion is spaced from and 5' of said first portion; and
one of the first and the second primers being labelled, with the other of said first and said second primers being anchored to a substrate;
subjecting a sample of nucleic acid comprising first and, complementary, second strands to an amplification reaction in the presence of said first and second oligonucleotide primers;
removing unreacted labeled primer; and
detecting the presence of amplified labeled nucleic acid captively anchored on said substrate.

28. A method of detecting the presence or absence of a mutation at a predetermined site in a predetermined nucleic acid sequence according to claim 27 wherein is used a first oligonucleotide primer such that the 5'-end region of said first portion contains at least part of said predetermined site.

29. A method according to claim 28 wherein is also used a second oligonucleotide primer combination with a first oligonucleotide primer such that the respective first portion of the predetermined nucleic acid sequence is from the normal form of the nucleic acid.

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30. A method according to any one of claims 27 to 29 wherein is used an amplification reaction with at least 15 amplification cycles.
- 5 31. A method according to any one of claims 27 to 30 which includes a preliminary amplification stage with a second oligonucleotide primer combination.
- 10 32. A kit of parts suitable for use in a method of detecting the presence or absence of a predetermined nucleic acid sequence in a sample of nucleic acid and comprising a detection system according to any one of claims 1 to 26.
- 15 33. A system according to claim 17 or claim 18 wherein said predetermined nucleic acid sequence includes base 20210 of the genomic DNA sequence encoding clotting factor II (prothrombin).
- 20 34. A system according to claim 33 when dependent on claim 18 wherein is included an oligonucleotide primer combination with first and second oligonucleotide primers having the sequences 5'-ATAGCACTGGGAGCATTGAGGCTC-3' and 5'-ATAGCACTGGGAGCATTGAGGCT-3', and 5'-TCTAGAAACAGTTGCCTGGC-3', respectively.
- 25



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(54) Title: NUCLEIC ACID SEQUENCE DETECTION (57) Abstract <p>The present invention provides a detection system suitable for use in a method of detecting the presence or absence of a predetermined nucleic acid sequence in a sample of nucleic acid. The system comprises a first oligonucleotide primer capable of annealing with a first strand of nucleic acid containing said predetermined nucleic acid sequence, such that a 3' end region of the first primer anneals with a first portion of said predetermined nucleic acid sequence; a second oligonucleotide primer having a 3' end region capable of annealing with a second strand of nucleic acid complementary to said first strand of nucleic acid such that a 3' end region of the second primer anneals with a portion of said second strand which portion is complementary to a second portion of said predetermined nucleic acid sequence, which second portion is spaced from end 5' of said first portion. One of the first and the second primers is labelled and the other is anchored to a substrate. Upon subjecting a sample of nucleic acid to an amplification reaction in the presence of said first and second oligonucleotide primers the presence of said predetermined nucleic acid sequence is indicated by the production of an amplified labelled nucleic acid product captively retained on said substrate.</p>		

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 11369 A (HOLMES MICHAEL JOHN; CEMU BIOTEKNIK (SE)) 4 October 1990	1-18, 21-23, 27-32
Y	see the whole document	19,20, 24-26, 33,34
Y	--- WO 95 29259 A (STICHTING CENTRAAL LAB ;VOORBERG JOHANNES JACOBUS (NL); MOURIK JAN) 2 November 1995	19,20
A	Primer 506-7 and Primer 506-8 see the whole document	1-18, 21-34
Y	--- WO 95 21938 A (RIJKS UNIVERSITEIT LEIDEN ;BERTINA ROGIER MARIA (NL); REITSMA PIET) 17 August 1995	19,20
A	Primer 9 see the whole document	1-18, 21-34

	-/--	

☒ Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PATENT ABSTRACTS OF JAPAN vol. 095, no. 010, 30 November 1995 & JP 07 184695 A (SANWA KAGAKU KENKYUSHO CO LTD), 25 July 1995, See Hepatitis C virus sense PCR detection primer P16 ---	24-26
Y A	EP 0 521 318 A (LUCKY LTD) 7 January 1993 Hepatitis C virus detection PCR primer A see the whole document ---	24-26 1-23, 27-34
A	WO 93 09250 A (ADELAIDE CHILDREN S HOSPITAL ;UNIV SOUTH AUSTRALIA (AU)) 13 May 1993 see the whole document ---	1-34
P,Y	BLOOD, vol. 88, no. 10, 15 November 1996, pages 3698-3703, XP002037583 POORT ET AL.: "A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis" cited in the application see primer Fig.2 see the whole document -----	33,34

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 97/00548

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9011369 A	04-10-90	AT 147791 T AU 646220 B AU 5272890 A CA 2050681 A DE 69029726 D EP 0464067 A JP 4505999 T US 5629158 A	15-02-97 17-02-94 22-10-90 23-09-90 27-02-97 08-01-92 22-10-92 13-05-97
WO 9529259 A	02-11-95	AU 2319495 A CA 2188463 A EP 0756638 A	16-11-95 02-11-95 05-02-97
WO 9521938 A	17-08-95	AU 1809195 A DE 696325 T EP 0696325 A ES 2090001 T FI 954855 A JP 8509383 T	29-08-95 29-08-96 14-02-96 16-10-96 17-11-95 08-10-96
EP 0521318 A	07-01-93	AU 659649 B AU 1902892 A BR 9206131 A CA 2111108 A CN 1068335 A GB 2272443 A,B JP 6508024 T WO 9222655 A KR 9615513 B	25-05-95 12-01-93 02-05-95 23-12-92 27-01-93 18-05-94 14-09-94 23-12-92 15-11-96
WO 9309250 A	13-05-93	AU 667846 B BR 9206705 A CA 2122450 A EP 0672173 A	18-04-96 21-11-95 13-05-93 20-09-95